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VE-cadherin is a critical endothelial regulator of TGF-β signalling

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VE-cadherin is an endothelial-specific transmembrane protein concentrated at cell-to-cell adherens junctions. Besides promoting cell adhesion and controlling vascular permeability, VE-cadherin transfers intracellular signals that contribute to vascular stabilization. However, the molecular mechanism by which VE-cadherin regulates vascular homoeostasis is still poorly understood. Here, we report that VE-cadherin expression and junctional clustering are required for optimal transforming growth factor-β (TGF-β) signalling in endothelial cells (ECs). TGF-β antiproliferative and antimigratory responses are increased in the presence of VE-cadherin. ECs lacking VE-cadherin are less responsive to TGF-β/ALK1- and TGF-β/ALK5-induced Smad phosphorylation and target gene transcription. VE-cadherin coimmunoprecipitates with all the components of the TGF-β receptor complex, TβRII, ALK1, ALK5 and endoglin. Clustered VE-cadherin recruits TβRII and may promote TGF-β signalling by enhancing TβRII/TβRI assembly into an active receptor complex. Taken together, our data indicate that VE-cadherin is a positive and EC-specific regulator of TGF-β signalling. This suggests that reduction or inactivation of VE-cadherin may contribute to progression of diseases where TGF-β signalling is impaired.

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Introduction

Transforming growth factor-β (TGF-β) is a multifunctional dimeric polypeptide growth factor that regulates proliferation, differentiation, migration, extracellular matrix production and survival of various cell types. TGF-β mediates its cellular effects through ligand-induced heteromeric receptor complexes of type II and type I transmembrane serine-threonine kinases. Activated type I receptors phosphorylate cytoplasmic receptor-associated Smad proteins (R-Smads). Phosphorylated R-Smads dissociate from the receptor and form a complex with the common mediator Smad4. This complex accumulates in the nucleus where it regulates transcription by interacting with many specific DNA-binding proteins (Attisano and Wrana, 2002; Derynck and Zhang, 2003).

In endothelial cells (ECs), the TGF-β type II receptor (TβRII) and two distinct TGF-β type I receptors (TβRIs), the EC-restricted ALK1 and the broadly expressed ALK5, are expressed (Oh *et al*, 2000; Goumans *et al*, 2002). ALK1 activation induces the phosphorylation of Smad1/5/8, whereas ALK5 promotes Smad2/3 phosphorylation. In addition, TGF-β also binds the co-receptor endoglin, a modulator of ALK1 and ALK5 signalling, highly expressed on rapidly proliferating ECs. In some experimental conditions, endoglin increases ALK1 signalling and promotes EC proliferation and angiogenesis (Goumans *et al*, 2002; Lebrin *et al*, 2004). In other experimental settings, lack of endoglin increases response to ALK1 and subsequently growth (Pece-Barbara *et al*, 2005). These conflicting results suggest that the cellular context may strongly influence the downstream effect of TGF-β activation in ECs.

Hereditary haemorrhagic telangiectasia (HHT) is an autosomal dominant vascular dysplasia characterized by epistaxis, telangiectases, pulmonary and cerebral vascular malformations and later in life by gastrointestinal bleeding. Two main genes are mutated in this disease, *ENG* in HHT1 and *ALK1* in HHT2 that account for about 80% of cases (Lesca *et al*, 2006; Prigoda *et al*, 2006), indicating that yet unidentified genes are also responsible for this disease.

Mice lacking either endoglin or ALK1 die *in utero* at embryonic day 10.0–10.5 of major defects in vascular and heart development (Bourdeau *et al*, 1999; Li *et al*, 1999; Arthur *et al*, 2000; Oh *et al*, 2000; Urness *et al*, 2000). The phenotype is characterized by haemorrhages in the yolk sac and embryo proper due to lack of maturation of the primitive vascular plexus with abnormal dilation of the lumen and vascular rupture. In the heart, the ECs of the endocardium fail to undergo mesenchymal transition required for their migration into the atrio-ventricular cushion. This defect strongly affects heart development and function (Bourdeau *et al*, 1999; Arthur *et al*, 2000; Sorensen *et al*, 2003).

The vascular phenotype of endoglin- and ALK1-deficient embryos presents striking similarities with that of embryos lacking proteins of endothelial cell-to-cell adherens junctions. These structures are formed by an endothelial-specific member of the cadherin family (VE-cadherin), which promotes

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homotypic cell–cell adhesion and which, through its cytoplasmic domain, links intracellular partners such as β-catenin, p120 and plakoglobin (Dejana, 2004; Gumbiner, 2005). Adherens junctions are required for EC stabilization and homoeostasis as they promote contact inhibition of growth and reduce cell sensitivity to apoptotic stimuli. Embryos lacking VE-cadherin die *in utero* by embryonic day 10.5 from alterations in the vascular development of the yolk sac and embryo. Vessels cannot undergo remodelling, are fragile and haemorrhagic, tend to grow in an irregular way and eventually regress (Carmeliet *et al*, 1999). Consistently, EC-specific inactivation of the β-catenin gene leads to embryonic death due to abnormal development of the vascular system with lacunae, enlarged and irregular lumen and multiple haemorrhages (Cattelino *et al*, 2003). Most strikingly, β-catenin-null endocardial cells fail to invade the atrio-ventricular cushion (Liebner *et al*, 2004) leading to a defect comparable to that described in endoglin/ALK1 knockout embryos. In adult mice, transplantation of VE-cadherin-null ECs leads to pathological features, such as the formation of large haemangiomas, abnormal EC growth and frequent bleeding (Zanetta *et al*, 2005).

The mechanism of action of VE-cadherin/β-catenin signalling is complex and several pathways may be affected by its inhibition (Dejana, 2004; Liebner *et al*, 2006). We found that VE-cadherin may associate with VEGFR-2 and reduce its proliferative signal, thus limiting EC growth and controlling vascular remodelling. This activity requires VE-cadherin clustering at junctions and association with DEP-1/CD148 phosphatase, responsible for VEGFR-2 receptor dephosphorylation (Lampugnani *et al*, 2003). However, the mechanism by which VE-cadherin signalling controls vascular stabilization is still poorly investigated. Previously, we demonstrated that β-catenin-null ECs/endocardial cells fail to respond to TGF-β and to undergo mesenchymal transition, suggesting the existence of a strict interplay between the VE-cadherin/β-catenin complex and TGF-β signalling (Liebner *et al*, 2004). In the present study, we investigated the role of VE-cadherin expression and clustering in TGF-β-induced biological responses. Our data demonstrate for the first time that VE-cadherin is a key positive regulator of TGF-β/ALK/Smad signalling in ECs.

VE-cadherin knockdown inhibited TGF-β-induced Smad1/5 and Smad2/3 phosphorylation, reduced expression of Smad target genes and counteracted the inhibitory effect of TGF-β on EC proliferation and migration. TGF-β stimulation induced TβRII association with VE-cadherin at cell-to-cell contacts where VE-cadherin clustering may promote TβRII/TβRI assembly and activation. Overall our data indicate that VE-cadherin participates in maximal activation of the TGF-β pathway, acting as a positive and EC-specific regulator of TGF-β response. This indicates that the response of ECs to growth factors is context dependent and that increased TGF-β signalling may contribute to VE-cadherin-dependent stabilization and remodelling of the vascular endothelium.

Results

VE-cadherin increases TGF-β/Smad-dependent transcription

To examine whether VE-cadherin has a regulatory role in TGF-β signalling in ECs, we used mouse embryonic stem cell-derived ECs, with homozygous null mutation of the VE-cadherin gene (VEC null) and reconstituted by retroviral transfer to express wild-type levels of VE-cadherin (VEC positive), as described previously (Balconi *et al*, 2000; Lampugnani *et al*, 2002, 2003; Iurlaro *et al*, 2004). We analysed these cells in a transcriptional response assay using a TGF-β-inducible reporter. The ALK5-dependent transcriptional reporter $(CAGA)_{12}$-luc (Dennler *et al*, 1998) was activated by TGF-β in VEC-positive cells; however, the stimulatory effect on the reporter was greatly reduced in the VEC-null cells (Figure 1A). To validate these findings in different cells, we repeated the reporter assays in mouse embryo-derived ECs (MEECs) from VE-cadherin knockout and wild-type embryos and in TGF-β responsive CHO cells transfected with VE-cadherin or control vector (Breviario *et al*, 1995). The presence of VE-cadherin led to higher TGF-β response in all three cell types (Figure 1A).

We next tested the effect of TGF-β on the expression of plasminogen activator inhibitor-1 (PAI-1), downstream of the ALK5 receptor and on the inhibitor of differentiation-1 (Id1), an ALK1-specific target gene (Goumans *et al*, 2002). Both genes were robustly upregulated in the VEC-positive cells compared with the null cells (Figure 1B).

These experiments indicate that VE-cadherin expression is a positive regulator of TGF-β-induced transcriptional responses in ECs.

VE-cadherin is essential for TGF-β-induced inhibition of EC cell growth and migration

To examine the effect of VE-cadherin on TGF-β-induced EC growth inhibition, we counted the number of viable cells present after 5 days of TGF-β treatment (5 ng/ml) by direct counting (Figure 2A). Consistent with previous results, VEC-null cells proliferate faster than VEC-positive cells and achieve higher cell densities (Lampugnani *et al*, 2003). However, TGF-β inhibitory effect on cell proliferation was consistently greater in VEC positive (50%) than in null cells (21%). We next measured the contribution of VE-cadherin to the TGF-β inhibition of migratory behaviour of human umbilical vein ECs (HUVECs), as TGF-β is known to tightly control EC migration during angiogenesis (Muller *et al*, 1987; Basson *et al*, 1992). VE-cadherin expression was ablated by siRNA (Supplementary Figure 1S) and migratory behaviour was assessed by wound assay and time-lapse microscopy (Figure 2B). Short time intervals (3–12 h) were selected, where the role of VE-cadherin in migration is not apparent. siRNA-mediated knockdown of VE-cadherin blocked the inhibitory effect of TGF-β on HUVEC migration. HUVEC transfected with siRNA for VE-cadherin, either untreated or TGF-β-treated, displayed a constant migration speed of 12 μm/h. Control siRNA-transfected cells also displayed a migration speed of 12 μm/h, but reduced by TGF-β to 8 μm/h.

We validated this observation in a different EC model system. VEC-null and -positive cells were subjected to a wound assay and migrated cells were visualized by crystal violet and analysed by contrast phase microscopy (Figure 2C). VEC-null cells exhibited a basally higher migratory behaviour than VEC-positive cells, in accordance with VE-cadherin ability to prevent cell detachment from a confluent monolayer (Navarro *et al*, 1995). More importantly, VEC-null cell motility was not inhibited by TGF-β, whereas that of VEC-positive cells was, indicating that VE-cadherin positively regulates TGF-β-mediated inhibition of EC migration. The difference in cell migration was found at

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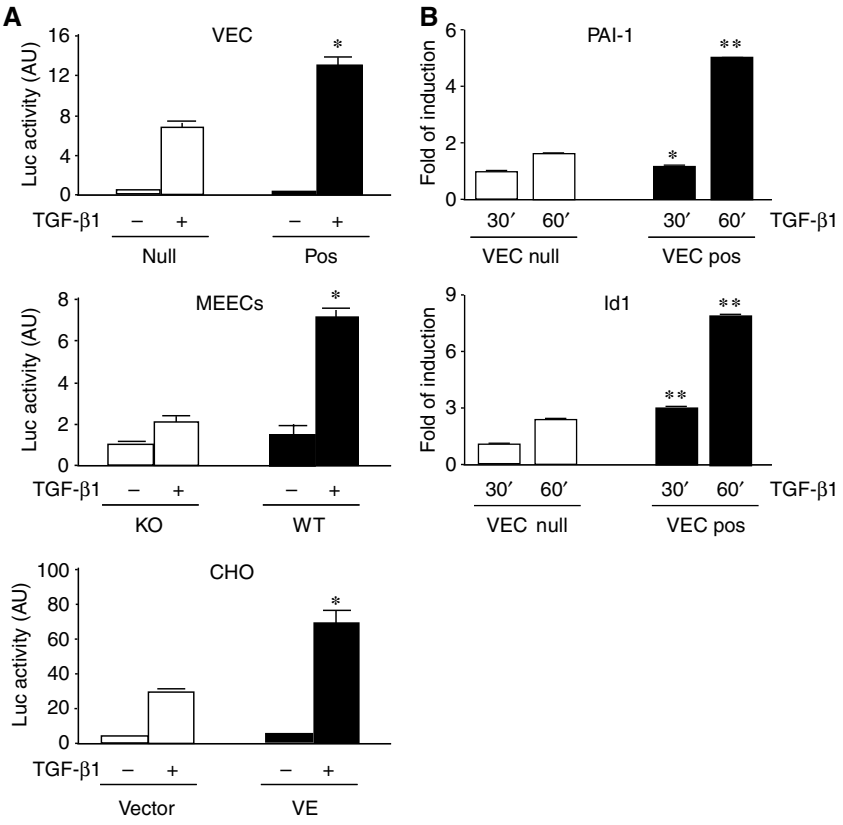

Figure 1 VE-cadherin enhances TGF-β-induced transcription. (**A**) VE-cadherin is important for optimal activation of TGF-β-induced reporter activity. VEC-null and -positive (Pos) cells, MEECs from VE-cadherin knockout (KO) and wild-type (WT) embryos, and CHO cells bearing a control vector (Vector) or VE-cadherin (VE) cDNA were transfected with a TGF-β-inducible reporter and stimulated with (+) or without (−) TGF-β1. Data are represented as means ± A.D. of six replicates from a representative experiment out of three performed. The statistical difference between the two cell lines is indicated (*$P<0.0001$). (**B**) Effect of VE-cadherin expression on TGF-β target genes PAI-1 and Id1. VEC-null (open bars) and -positive (closed bars) cells were stimulated with TGF-β1 (2.5 ng/ml) for the indicated times. Fold changes in gene expression were calculated using the $2^{-\Delta\Delta C_t}$ method. Error bars represent s.e. ($n=3$) from a representative experiment out of two performed. The statistical difference between the two cell lines is indicated (*$P=0.003$ and **$P<0.001$).

time intervals (12 and 24 h) where TGF-β has no antiproliferative activities on VEC cells (not shown), suggesting that VE-cadherin specifically regulates TGF-β effect on EC motility. Taken together, our results suggest that VE-cadherin expression is critical for TGF-β inhibitory activity of EC proliferation and migration.

VE-cadherin is necessary for efficient ALK1- and ALK5-dependent Smad phosphorylation in TGF-β-treated ECs

TGF-β elicits biological responses in ECs through two distinct type I receptors, ALK1 and ALK5, which induce the phosphorylation of Smad1/5/8 and Smad2/3, respectively, and their nuclear translocation (Goumans *et al*, 2002, 2003). To assess at which level of the signalling cascade VE-cadherin impacts TGF-β pathway, we analysed whether VE-cadherin-positive effect on TGF-β-induced response correlated with increased phosphorylation of TGF-β-dependent Smads. In time-dependent experiments, Smad1/5 phosphorylation was more transient than that of Smad2/3 (Figure 3). In VEC-positive cells peak levels for Smad1/5 and Smad2/3 phosphorylation were reached after 45 min of stimulation with TGF-β1 and were higher (2–2.5-fold) than in null cells, whereas total Smad levels were substantially unchanged (Figure 3). Shortly after TGF-β stimulation the onset and intensity of both Smad1/5 and Smad2/3 phosphorylation was increased, whereas at later time points only the duration of Smad2/3 phosphorylation was prolonged by VE-cadherin expression (Figure 3). In dose-dependent experiments, VEC-null cells showed 3- to 4-fold decrease in magnitude of both Smad1/5 and Smad2/3 phosphorylation, at equivalent TGF-β concentrations, indicating a significantly lower sensitivity of these cells to TGF-β (Figure 4). The enhanced TGF-β response of cells expressing VE-cadherin was not due to increased TβRII, ALK5 and ALK1 expression as shown by the analysis of TGF-β receptor levels (Supplementary Figure 2S).

To substantiate these findings in a different EC system, we repeated the analysis of Smad phosphorylation in HUVEC transfected with VE-cadherin or negative control siRNA. Silencing VE-cadherin expression resulted in a significant decline in TGF-β ability to induce ALK1- and ALK5-dependent Smad phosphorylation in the presence of unchanged Smad total levels (Supplementary Figure 3S). This effect was less marked than in VEC-null cells likely due to incomplete VE-cadherin knockdown (~80%) achieved by siRNA interference in these cells.

Taken together, these results indicate that VE-cadherin increases TGF-β/ALK1- and TGF-β/ALK5-mediated responses in ECs by acting upstream of Smad phosphorylation.

VE-cadherin forms a complex with TβRs

In ECs, signalling by TGF-β is initiated by binding to TβRII followed by recruitment of TβRI complexes and subsequent

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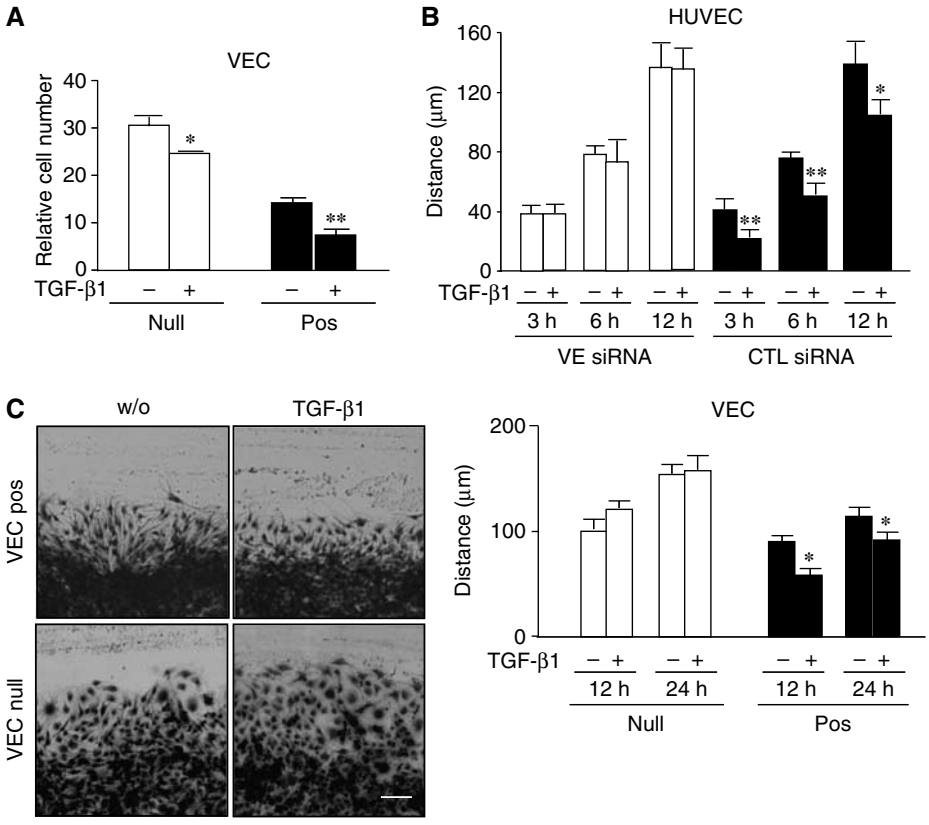

Figure 2 VE-cadherin potentiates TGF-β inhibitory effect on EC growth and migration. (**A**) VEC-null cells are less sensitive to TGF-β-induced growth inhibition than VEC-positive cells. VEC-null (Null) and -positive (Pos) cells were stimulated with (+) or without (−) TGF-β1 for 5 days. The number of viable cells was determined by Trypan blue and haemocytometer counting. Columns are means ± A.D. of triplicate from a representative experiment. $*P = 0.02$; $**P = 0.002$. (**B**) siRNA-mediated knockdown of VE-cadherin blocks TGF-β-induced inhibition of wound repair in HUVECs. Cells transfected with VE-cadherin (VE) or control siRNA (CTL) were starved, wounded and treated with (+) or without (−) TGF-β1. Migration of cells out of the wounded edge was recorded at the indicated times. The mean migrated distance and A.D. ($n = 6$) of a representative experiment is shown. $*P = 0.002$; $**P < 0.001$. (**C**) Lack of VE-cadherin blocks TGF-β-induced inhibition of wound repair in VEC cells. EC monolayers were wounded and stimulated with (+) or without (−) TGF-β1. At the indicated times the cells were fixed and stained with crystal violet. The migration of the cell front was measured by ImageJ software. The mean migrated distance and A.D. ($n = 9$) of a representative experiment out of three performed are shown. $*P < 0.001$. Left panels show the migrating front 12 h after wounding the EC monolayer. Scale bar = 40 μm.

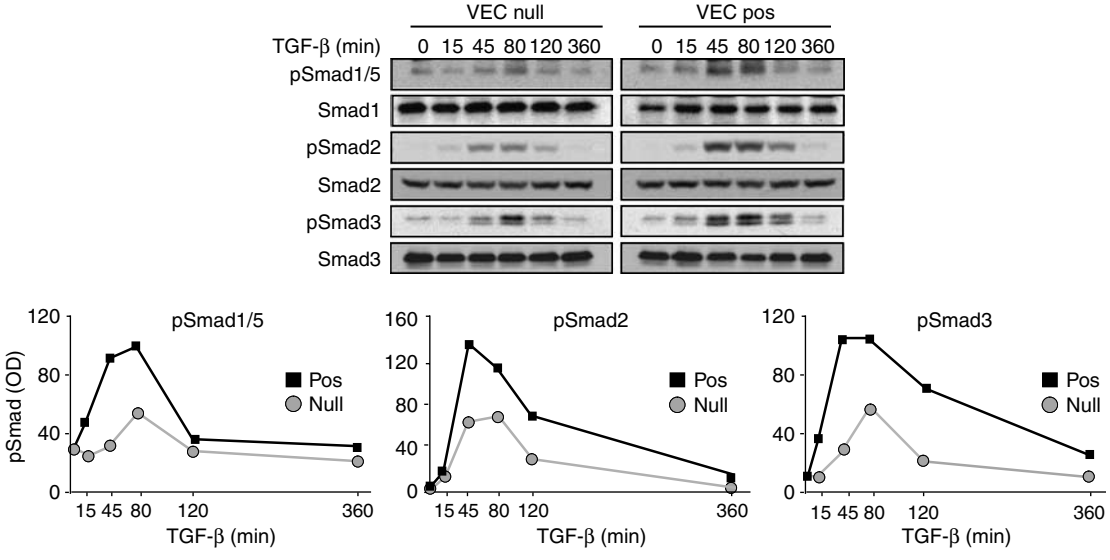

Figure 3 More rapid kinetics of TGF-β-induced Smad phosphorylation in VE-cadherin-expressing ECs. VEC cells were serum starved and stimulated with TGF-β1 (0.5 ng/ml) for the indicated times. Whole cell extracts were fractionated by 10% SDS–PAGE and blotted with phospho-specific Smad1/5, Smad2 and Smad3 antibodies. Total Smad1/2/3 levels were also probed for loading control. Lower graphs show the intensities of pSmad bands quantified by densitometry scan, corrected for background and plotted as optical density (OD). These results were confirmed by Odyssey Infrared Imaging System Analysis (data not shown).

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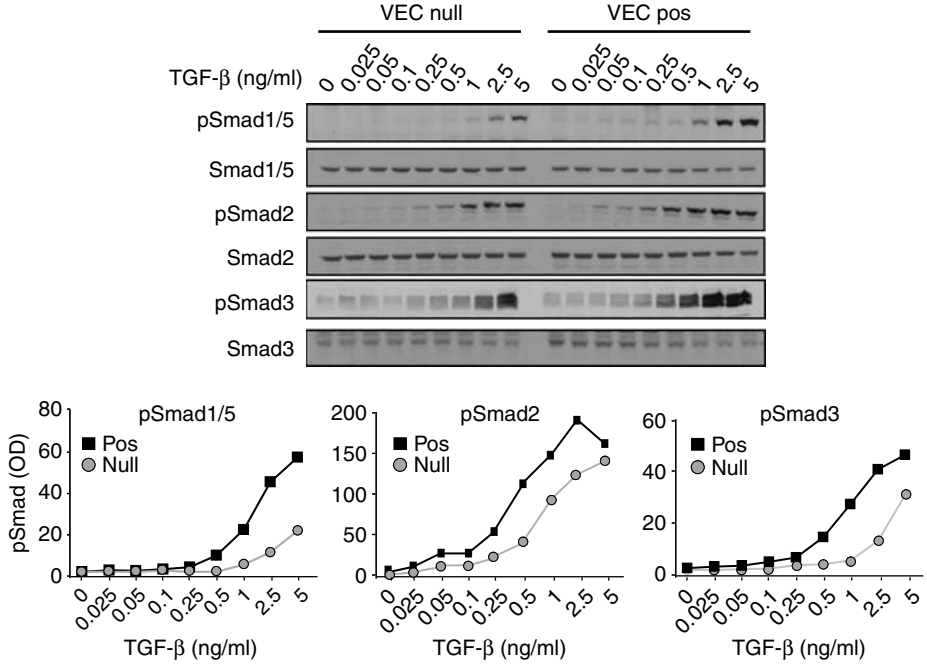

Figure 4 Dose–response of TGF-β-induced Smad phosphorylation is enhanced by VE-cadherin. Serum-starved VEC cells were stimulated with the indicated concentrations of TGF-β1 for 1 h. Whole cell extracts were blotted and quantified by densitometry scan. Lower graphs show the intensities of pSmad bands corrected for background and plotted as optical density (OD). The experiments reported in this figure were performed three times with comparable results.

Smad phosphorylation (Goumans *et al*, 2003). As VE-cadherin increases TGF-β signalling upstream of Smad phosphorylation, we hypothesized that it might do so through physical association with the TGF-β receptor complex.

To test this hypothesis, we immunoprecipitated TβRs from COS-1 cells co-transfected with VE-cadherin and either TβRII, ALK5 or ALK1. In the immunoprecipitates, we not only recovered the components of the TGF-β receptor complex but also VE-cadherin, indicating that VE-cadherin interacts with TβRII, ALK5 and ALK1 in the absence of exogenously added ligand (Figure 5A).

We confirmed previous results (Barbara *et al*, 1999; Abdalla *et al*, 2000; Pece-Barbara *et al*, 2005) that endoglin is present in the TGF-β receptor complex and interacts with TβRII, ALK5 and ALK1 (Figure 5A). We assessed whether endoglin might associate with VE-cadherin and modulate its binding to TβRs. We report here that endoglin binds VE-cadherin efficiently (Supplementary Figure 4S, panel A) but is not required for the VE-cadherin/TβRs complex to form, although it is most likely present in the complex *in vivo* (Supplementary Figure 4S, panel B). To ascertain that the analysed VE-cadherin/TβR complexes from transfected COS-1 cells occurred at the cell surface and not in internal compartments, both *in vivo*-formed immune complexes analysis and surface biotinylation and coimmunoprecipitation analysis were used. Using both approaches, VE-cadherin was recovered from TβRII- and TβRI-containing immunoprecipitates, indicating that the association between VE-cadherin and ectopically expressed TβRs occurs between cell surface exposed molecules in transfected COS-1 cells (Supplementary Figure 5S).

Next, we examined endogenous TβRII using surface biotinylation of VEC-positive cells followed by immunoprecipitation with anti-TβRII and detection using streptavidin. We found that a surface protein comigrating with VE-cadherin was coimmunoprecipitated by TβRII (Figure 5B). To confirm the association of endogenous TβRII and VE-cadherin and assess whether their binding was TGF-β dependent, we carried out immunoprecipitation and immunoblotting of TGF-β-treated VEC-positive cells. We found that endogenous TβRII interacts with VE-cadherin and that this interaction slowly increases between 15 and 45 min of ligand stimulation (Figure 5C) and is still sustained at 2 h (data not shown). Finally, we immunoprecipitated endogenous ALK5 and found that VE-cadherin is an interacting partner also of this receptor in TGF-β-stimulated cells (Figure 5D).

Disruption of VE-cadherin clustering inhibits TGF-β-dependent Smad phosphorylation

Proper clustering of VE-cadherin at interendothelial junctions is required for optimal inhibition of VEGFR-2 phosphorylation and mitogenic signalling (Lampugnani *et al*, 2003). To test whether VE-cadherin clustering was essential to exert its positive effect on TGF-β signalling, we first assessed whether transiently expressed TβRII codistributed with VE-cadherin at junctions in cultured VEC-positive cells by indirect immunofluorescence and confocal microscopy (Figure 6A). VE-cadherin staining was mostly localized at discrete spots of adjacent plasma membranes, as expected for a junctional protein. TβRII displayed a vesicular and cell surface localization. Consistent with a possible localization at intercellular adherens junctions, TβRII showed colocalization with VE-cadherin at cell-to-cell contacts (Figure 6A). The quantification of the average number of colocalization events at the plasma membrane showed weak codistribution in the untreated state followed by a two-fold increase upon stimulation with 2 ng/ml TGF-β for 90 min (Figure 6B). We examined

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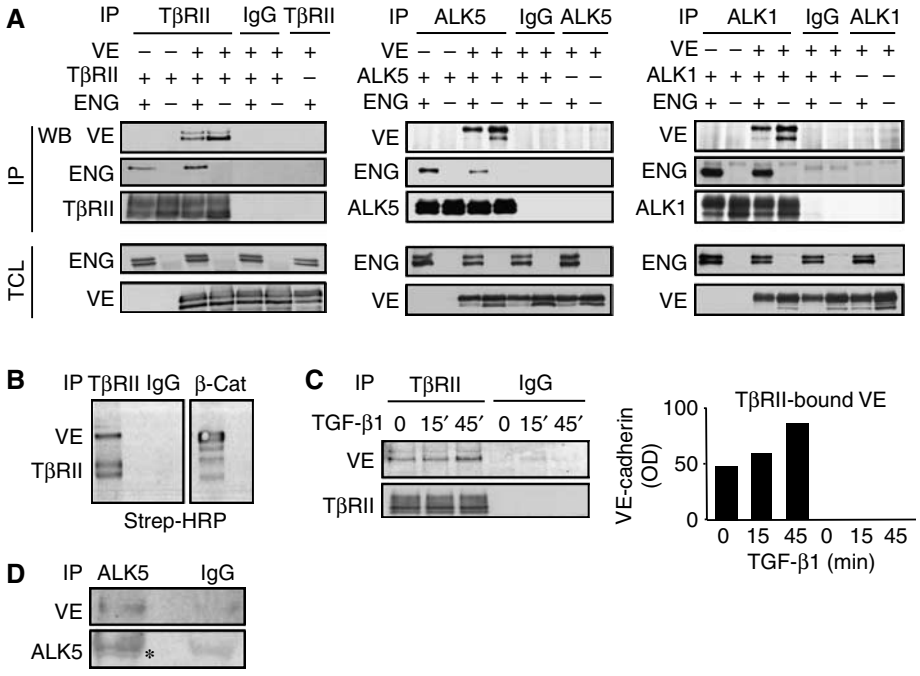

Figure 5 VE-cadherin associates with the TβR complex. (**A**) VE-cadherin associates with transiently expressed TβRs in COS-1 cells. Cells were transfected with the indicated constructs, immunoprecipitated (IP) with specific antibodies (TβRII, ALK5 and ALK1) or isotype control IgG, and blotted (WB) as indicated. Total cell lysates (TCLs) were loaded to control for transfection efficiency. Apparent decrease in VE-cadherin/TβRs association is due to a lower TβR constructs expression induced by endoglin. ENG, endoglin; VE, VE-cadherin. (**B**) Surface interaction of endogenous TβRII and VE-cadherin. VEC positive cells were serum starved, sulfo-NHS-biotinylated on ice, rinsed, solubilized, immunoprecipitated with anti-TβRII or isotype control IgG and probed with streptavidin-horseradish peroxidase (Strep-HRP). Cell lysates immunoprecipitated with β-catenin antibody (β-cat) were used to control for biotinylated VE-cadherin. (**C**) Ligand-dependent interaction of VE-cadherin with endogenous TβRII. VEC-positive cells were serum starved and stimulated with TGF-β1 (5 ng/ml) for the indicated time intervals. Cell extracts were immunoprecipitated (IP) with TβRII antibodies or control isotype IgG and blotted with VE-cadherin (VE) or TβRII antibody. The intensities of TβRII-bound VE signals were quantitated by densitometry scan and are represented as optical density (OD). (**D**) Endogenous ALK5/VE-cadherin association. VEC-positive cells stimulated with TGF-β1 (5 ng/ml) for 45 min, followed by treatment with cross-linker for 20 min, were immunoprecipitated (IP) with ALK5 antibody or control isotype IgG and blotted with VE-cadherin (VE) or ALK5 antibody. *ALK5 doublet.

the cell surface distribution of endogenous TβRII/TβRI complexes by *in vivo* treatment with biotinylated TGF-β1 and FITC-conjugated avidin followed by immunofluorescence confocal microscopy. After stimulation of cells with biotinylated TGF-β1, cell surface TβRII/TβRI active signalling complexes localized to puncta unevenly distributed over the plasma membrane. A subset of these active TGF-β receptor complexes concentrated at cell contacts where they localized with VE-cadherin (Figure 6C).

Thus, biochemical and morphological data indicating ligand-dependent association and colocalization of endogenous TβRs with VE-cadherin suggest that VE-cadherin recruits TβRs at cell-to-cell contacts during signalling.

To test whether proper clustering of VE-cadherin at interendothelial junctions was required for its effect on TGF-β signalling, we treated confluent monolayers of VEC-positive cells with 5 mM EGTA during TGF-β stimulation to fully disrupt VE-cadherin clustering at cell-to-cell contacts (Supplementary Figure 6S). Only low levels of phosphorylated Smad2 and Smad3 were detectable in EGTA-treated VEC-positive cells challenged with TGF-β, in contrast to control cells treated with TGF-β in the absence of EGTA, suggesting that a robust activation of Smad signalling in endothelial monolayers requires correct homophilic interactions of VE-cadherin molecules. We further confirmed this finding by a monoclonal antibody (BV9) known to block VE-cadherin-adhesive properties in a highly specific way (Corada *et al*, 1999, 2001). We treated HUVEC confluent monolayers with BV9 to dismantle VE-cadherin from intercellular junctions and induce its redistribution on the cell membrane (Supplementary Figure 7S). Consistent with the Ca^{2+} chelation results, we observed a substantial decrease of TGF-β-dependent Smad3 phosphorylation in confluent HUVECs treated with BV9 compared with isotype control-treated cells (Figure 7). Overall, these data indicate that VE-cadherin clustering at interendothelial junctions is required for efficient TGF-β-induced Smad phosphorylation.

Clustered VE-cadherin binds TβRs and promotes their assembly into an active receptor complex

TβRs exist as multimeric complexes on the cell surface and their assembly has a strong impact on mediating and regulating intracellular signals (Shi and Massague, 2003). To test whether VE-cadherin may induce complex formation between TβRII and TGF-β type I receptors, we immunoprecipitated endogenous ALK5 or ALK1 from VEC-positive and -null cells treated with 5 ng/ml TGF-β for 45 min, and probed the immune complexes for the associated TβRII (Figure 8). As expected, TβRII was found to form a complex with ALK1 and ALK5 in the presence of ligand. However, in contrast to ALK5, we observed a consistently weaker ligand-induced association of ALK1 and TβRII receptors. This effect, in the presence of signalling, as judged by Smad1/5 phosphorylation (data not shown), is possibly due to a lower ALK1 relative

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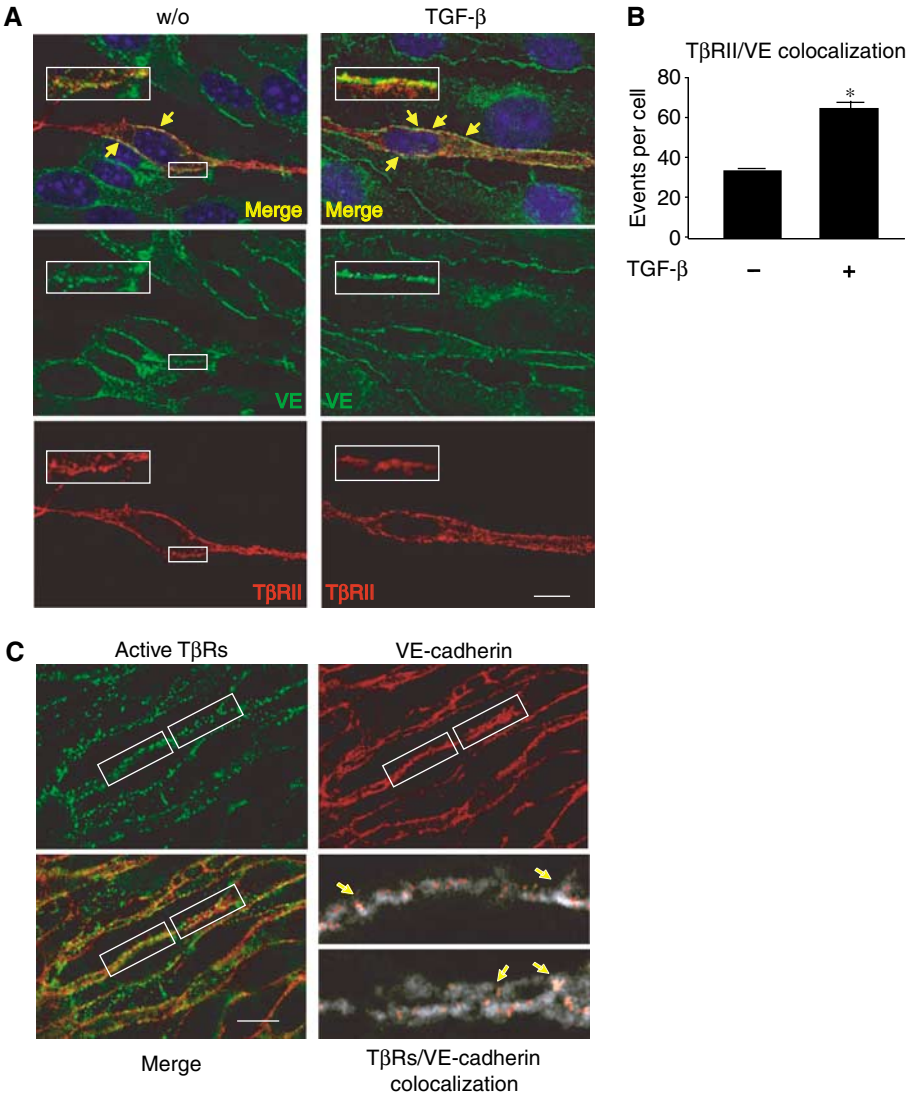

Figure 6 VE-cadherin colocalizes with TβRs at cell contacts. (**A**) TGF-β-induced colocalization of transiently expressed TβRII and VE-cadherin at cell–cell junctions. VEC-positive cells transfected with TβRII, and stimulated with (TGF-β) or without (w/o) TGF-β1 (2 ng/ml), were stained with the polyclonal antibody anti-TβRII (red), and the monoclonal anti-VE-cadherin (green). Overlay of the two images is shown (merge), with colocalization appearing as yellow. Areas marked by a rectangle are enlarged and shown as insets. Scale bar = 10 μm. **(B)** Quantification of colocalization between VE-cadherin and TβRII. Colocalization events per cell were acquired by immunofluorescence confocal analysis and quantitated using the ImageJ colocalization plug-in. Values are displayed as means ± s.d. of triplicates from one representative experiment. $^{*}P = 0.007$. **(C)** VE-cadherin colocalizes with endogenous TβRs. Serum-starved VEC-positive cells were incubated with biotinylated TGF-β1 and stained *in vivo* with FITC-conjugated avidin to detect the endogenous TβRII/TβRI active complexes (green). The cells were fixed and stained with the monoclonal VE-cadherin antibody (red). Overlay of the two images (merge) is shown. In the bottom right panels (magnification of the boxed areas) the arrows indicate TβRs/VE-cadherin colocalization (orange dots) set upon the VE-cadherin background (grey). Scale bar = 10 μm.

expression and/or affinity of the ALK1 antibody. Importantly, however, analysis of ALK5/TβRII and ALK1/TβRII complexes revealed that they were increased by VE-cadherin (Figure 8). Taken together, these data show that clustered VE-cadherin may enhance cellular responses to TGF-β by facilitating ligand-dependent assembly of TβRs into an active receptor complex, thus regulating their ability to induce R-Smad phosphorylation and signal propagation.

Allantoises from VE-cadherin-null embryos exhibit reduced TGF-β/Smad signalling

To test the relevance of the *in vitro* observations in an *ex vivo* model of vasculogenesis and angiogenesis, we used the mouse allantois organ culture model (Drake and Fleming, 2000; Downs *et al*, 2001). The allantois is an extraembryonic tissue undergoing extensive neovascularization in the developing mouse embryo at 8.5 d.p.c. (Argraves *et al*, 2002). When explanted, 8.5 d.p.c. allantoises display a highly branched network of capillary-like vessels after 24 h of culture; this property was much reduced in allantoises derived from VE-cadherin knockout ($VE^{-/-}$) embryos (Crosby *et al*, 2005). To test whether the loss of VE-cadherin might impair TGF-β signalling, we investigated by immunofluorescence and confocal microscopy the nuclear localization of ALK5-dependent Smads in capillary-like vessels from $VE^{-/-}$ and $VE^{+/+}$ allantoises. Consistent with the abundant

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expression of TGF-β and TβRs in developing allantois (Jonker and Arthur, 2002), pSmad nuclear localization in ECs was much increased upon stimulation of starved allantoises with TGF-β (Supplementary Figure 8S). However, we were able to detect a consistent and significant decrease of pSmad2/3 (27%) nuclear localization in TGF-β-treated $VE^{-/-}$ vs $VE^{+/+}$ vessels (Figure 9), implying a role for VE-cadherin as positive regulator of TGF-β signalling levels also in this experimental setting. Together with the *in vitro* studies, these data implicate adherens junction signalling as a significant contributor to TGF-β-induced maturation of blood vessels *in vivo*.

Discussion

In this study, we describe a novel pathway regulating TGF-β signalling in the endothelium. We found that the expression of the EC-specific adherens junction protein VE-cadherin enhances TGF-β-induced biological responses in ECs.

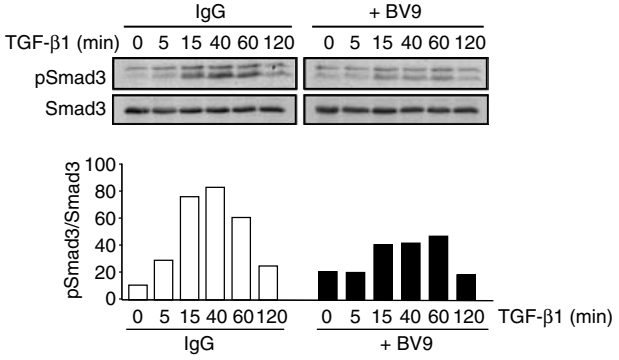

Figure 7 Dismantling of VE-cadherin clusters at endothelial contacts inhibits Smad3 phosphorylation. HUVECs grown to confluence were serum starved, incubated for 5 h with VE-cadherin blocking mAb (BV9), or isotype control IgG, and then treated with TGF-β1 for the indicated time intervals. Total cell extracts were blotted with anti-pSmad3 or Smad3 antibodies (upper panels), quantitated by densitometry scan, corrected for background and plotted as pSmad3/Smad3 ratio (lower panel).

Several lines of evidence support this conclusion. First, EC lines from VE-cadherin-null mice or primary ECs treated with VE-cadherin siRNA show attenuated TGF-β-induced transcriptional, antiproliferative and antimigratory responses even when expressing normal TGF-β receptors and Smad complement. Second, VE-cadherin is required for efficient ALK-dependent Smad phosphorylation as shown by faster and more potent activation of Smad1/5 and Smad2/3 signalling and more sustained propagation of Smad2/3 signals over time in VE-cadherin-expressing ECs. Third, specific inhibition of VE-cadherin clustering in endothelial monolayers results in decreased TGF-β/Smad signalling. When junctions are dismantled by Ca^{2+} depletion or by VE-cadherin blocking antibodies, the TGF-β/Smad phosphorylation is largely attenuated clearly demonstrating that TGF-β signalling in ECs is critically dependent on proper VE-cadherin engagement at adherens junctions.

We found that cell surface TβRII codistributes with VE-cadherin at intercellular contacts and that TGF-β increases TβRII accumulation at adherens junctions (Figure 6). Based upon previous studies showing that TβRII can be recruited into tight junctions through binding to the structural component Par6 (Ozdamar *et al*, 2005), and the observed physical interaction of VE-cadherin with TβRII independently of the TGF-β type I receptors and vice versa (Figure 5), we suggest that at junctional contacts VE-cadherin favours the reciprocal association of TβRII and TGF-β type I receptors in the presence of ligand. Indeed, the association of TβRII with ALK1 and ALK5 is increased by VE-cadherin explaining the increment of Smad1/5 and Smad2/3 phosphorylation and signalling.

Previous studies have shown that TGF-β/ALK5/Smad2/3 pathway leads to inhibition of cell migration and proliferation, whereas signalling through TGF-β/ALK1/Smad1/5/8 can promote cell growth and motility (Goumans *et al*, 2002, 2003). In our experimental setting, the net downstream effect of VE-cadherin-mediated increment in TGF-β signalling is inhibition of cell growth and motility. This was unexpected

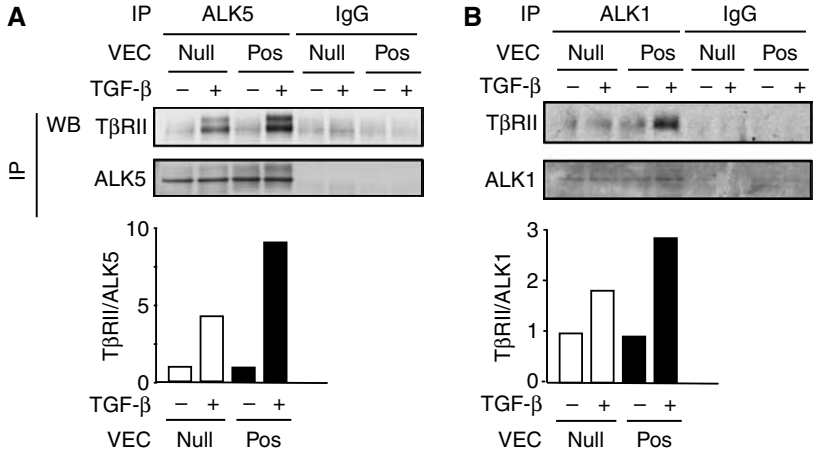

Figure 8 TGF-β-mediated assembly of TβRII/TβRI heteromers is increased by VE-cadherin. (**A**) TβRII/ALK5 complexes are enhanced in TGF-β-treated VEC-positive cells. Lysates from VEC-null (Null) and -positive (Pos) cells, unstimulated (−) or treated (+) with TGF-β1 (5 ng/ml) for 45 min, were subjected to immunoprecipitation (IP) with a protein A-sepharose-conjugated ALK5 antibody or isotype control IgG, followed by immunoblotting (WB) with TβRII and ALK5 antibody, as control for immunoprecipitation efficiency. Membranes were quantitated by densitometry scan, corrected for background and plotted as TβRII/ALK5 ratio (lower panel). (**B**) Increased TβRII/ALK1 complexes in TGF-β-treated VEC-positive cells. Cells were treated as described in (A) and ALK1/TβRII association was detected by immunoprecipitation (IP) with a protein A-sepharose-conjugated ALK1 antibody, followed by immunoblotting (WB) with TβRII and ALK1 antibody. Membranes were quantitated as described in (A) (lower panel).

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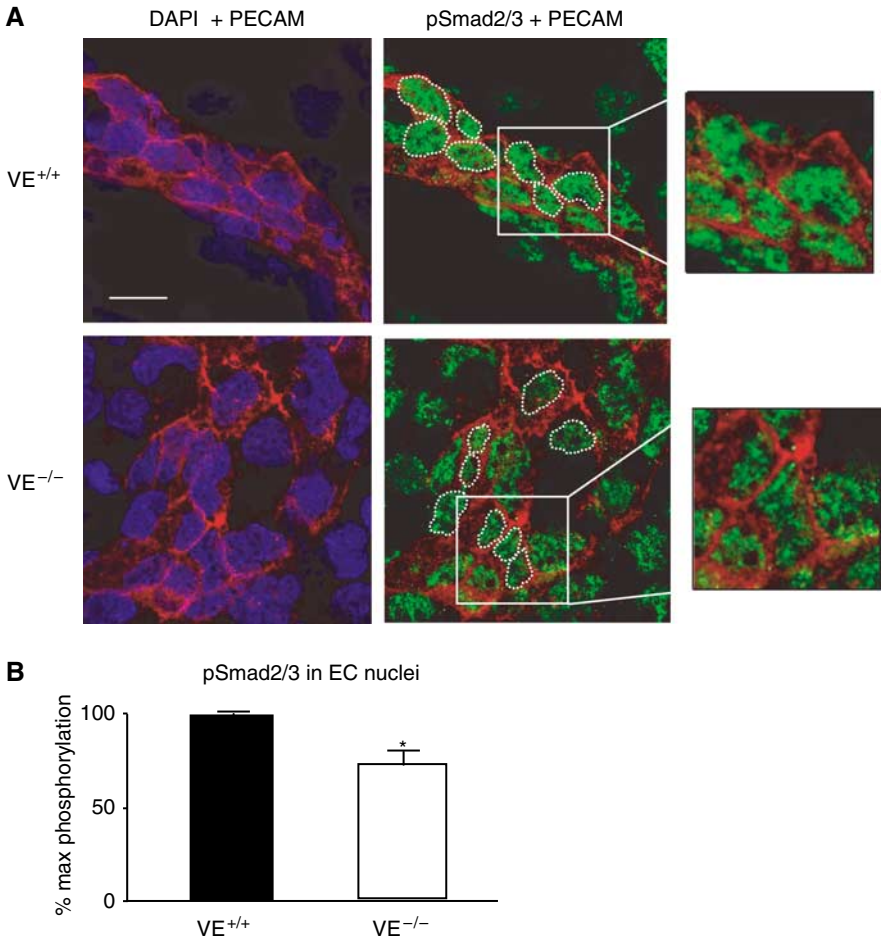

Figure 9 Loss of VE-cadherin reduces nuclear pSmad2/3 levels in TGF-β-treated allantois explants. (**A**) TGF-β-induced pSmad2/3 nuclear localization is reduced in VE-cadherin-null vessels. E8.5 allantoises from wild-type ($VE^{+/+}$) and VE-cadherin null ($VE^{-/-}$) mouse embryos were cultured *ex vivo* as described in Supplementary data. Localization of pSmad2/3 in ECs was visualized with antibodies to pSmad2/3 (green) and PECAM (red). Overlay of the two images is shown. Endothelial nuclei were visualized with DAPI (blue) and PECAM (red). Dotted lines mark endothelial nuclei. Areas marked by a rectangle are enlarged and shown as insets. Scale bar = 20 μm. (**B**) Quantification of endothelial pSmad2/3 signal from confocal microscopy images. Data are expressed as % decrease in phosphorylation compared with $VE^{+/+}$ cells referred as 100% (mean ± A.D. of 50 endothelial nuclei scored from three to four embryos per each genotype). $^{*}P = 0.0001$.

as not only ALK5 but also ALK1 receptor was activated. However, we observed that Smad2/3 activation was more persistent than that of Smad1/5 in the presence of VE-cadherin, indicating that at later times of TGF-β stimulation junctional VE-cadherin might tip the balance between these two pathways in favour of ALK5 signalling. As VE-cadherin associates with ALK1, which is also a signalling receptor for bone morphogenetic protein (BMP)-9 and -10, two cytokines with strong antiangiogenic activities in ECs (David *et al*, 2007; Scharpfenecker *et al*, 2007), VE-cadherin may have similar functions in BMP pathways although further studies are required to clarify this aspect.

The clinical manifestations and symptoms of HHT vary greatly even within a family, suggesting a potential role for modifier genes in the progression of this pathology. At this stage, a connection between the observations reported here and HHT is only speculative. However, it is conceivable that conditions which impair VE-cadherin expression or clustering at junctions may attenuate TGF-β signalling. VE-cadherin activity is strongly downregulated by inflammatory cytokines or agents that increase permeability such as histamine, thrombin or VEGF (Dejana, 2004). It is therefore tempting to speculate that when players of TGF-β signalling, such as endoglin and ALK1, are reduced, as in HHT, a further impairment of TGF-β signalling by a local reduction of VE-cadherin may cause vascular fragility and haemorrhages. It is interesting that VE-cadherin-2, a member of the protocadherin family (Telo *et al*, 1998), was reported to be downregulated in HHT1 and HHT2 blood outgrowth ECs (Fernandez-Lopez *et al*, 2007).

The role of VE-cadherin expression and clustering in the modulation of growth factor receptor signalling was previously described for VEGFR-2 (Lampugnani *et al*, 2003). In this case however, and in contrast to TGF-β receptors, the association of VE-cadherin with the receptor caused a decrease in its tyrosine phosphorylation and proliferative signals. Inhibition of VEGFR-2 signalling by VE-cadherin is mediated by the phosphatase DEP-1/CD148 which is co-clustered at junctions, dephosphorylates the receptor and limits its internalization and signalling (Lampugnani *et al*, 2006). In contrast to VEGFR-2, it is likely that the action of VE-cadherin on TGF-β pathway relies on the capacity of the junctional protein to associate with all the components of the TGF-β receptor complex enhancing, in this way, the interaction between TβRII and TGF-β type I receptors and increasing the downstream signalling. A general concept that may

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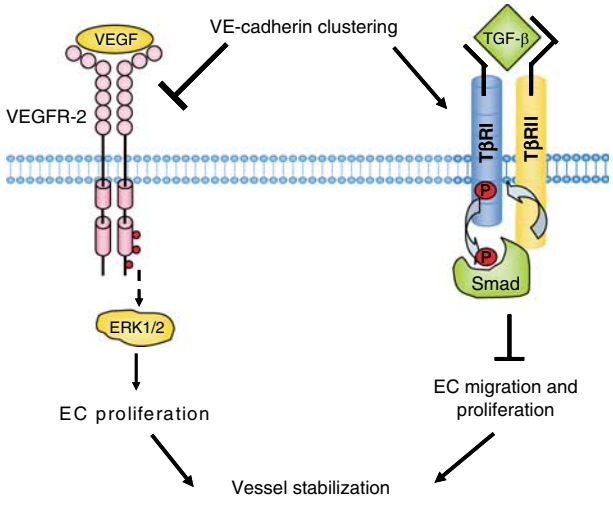

Figure 10 Proposed model of the role of VE-cadherin in quiescent endothelium. VE-cadherin regulates VEGF/ERK1/2 and TGF-β/Smad signalling pathways in opposite ways and with identical biological outcome in ECs. The formation of the VE-cadherin–VEGFR-2 complex limits cell proliferation by preventing ERK1/2 MAPK activation (Lampugnani *et al*, 2006). The formation of VE-cadherin–TβR complex promotes TGF-β-induced Smad phosphorylation leading to vascular stabilization.

accommodate these observations is that VE-cadherin acts by inducing vascular stabilization. Through the clustering of this protein, ECs are able to sense the presence of other adjoining identical cells and react by limiting their growth and migration. This action may be exerted by VE-cadherin recruiting growth factor receptors and modulating their activity. These opposing functions of VE-cadherin, inhibitory on VEGFR-2 and stimulatory on TβRII/ALK signalling, support a model (Figure 10) whereby EC response to growth factors is context dependent and ECs activated by the same growth factor may respond in opposite ways whether they are confluent and VE-cadherin organized at junctions or not. This model has important biological implications as it describes the quiescent phenotype of ECs as an active state mediated by continuous inhibition of growth and enhancement of stabilization signals.

Materials and methods

Transient transfections and transcriptional reporter assays

Transient transfections and reporter assays were performed as previously described (Felici *et al*, 2003). In all the reporter assays, a β-galactosidase expression vector was used as an internal control to correct for transfection efficiency.

Quantitative real-time RT–PCR

Total RNA from VEC cells was isolated with the RNeasy mini kit (Quiagen Inc.). cDNA was synthesized, amplified and analysed on an ABI PRISM®7900 as previously described (Spagnuolo *et al*, 2004).

EC proliferation assay

VEC cells were seeded in starvation medium (DMEM, 2% FCS) at a density of 5×10^4 cells per well in 24-well plates. After 12 h, TGF-β1 was added at 5 ng/ml. At the indicated time of culture, cells were trypsinized and the number of viable cells, in triplicate wells, was determined by Trypan blue and haemocytometer counting.

VE-cadherin gene silencing by siRNA

For VE-cadherin silencing, ON-TARGETplus SMARTpool duplex siRNAs were used as described in Supplementary data.

Wound-healing migration assay

EC monolayers were wounded with a yellow tip to make a ~100 μm scratch. The wound-induced cell migration in the presence of TGF-β1 (5 ng/ml) was followed by time-lapse microscopy (HUVECs) or crystal violet staining (0.5% crystal violet in 20% methanol) and phase contrast microscopy (VEC cells). The migration of the cell front was analysed by ImageJ software.

Immunoprecipitation and western blot analysis

Immunoprecipitation of total TβRs and cell surface biotinylated TGF-β receptors were performed as described previously (Felici *et al*, 2003; Pece-Barbara *et al*, 2005) and in Supplementary data.

Immunofluorescence microscopy

Immunofluorescence analysis of VE-cadherin and ectopically expressed or endogenous TGF-β receptors was performed as described previously (Felici *et al*, 2003; Lampugnani *et al*, 2006) and in Supplementary data.

VE-cadherin declustering

For disruption of VE-cadherin clustering, EC monolayers were treated as described previously (Corada *et al*, 1999, 2001) and in Supplementary data.

Allantois culture

Allantoises were dissected from 8.5 d.p.c. mouse embryos and cultured as described previously (Argraves *et al*, 2002) and in Supplementary data.

Statistical analysis

A Student's two-tailed non-paired *t*-test was used to determine the statistical significance. $P<0.05$ was considered statistically significant.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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